Characterization of the interferon pathway in the teleost fish gonad against the vertically transmitted viral nervous necrosis virus

Running title. IFN pathway is differently regulated in the gonad

Contents Category. RNA virus

Yulema Valero[1], Patricia Morcillo[2], José Meseguer[2], Francesco Buonocore[3], María A. Esteban[2], Elena Chaves-Pozo[1], Alberto Cuesta[2]*

[1]*Centro Oceanográfico de Murcia*, *Instituto Español de Oceanografía* (IEO), Carretera de la Azohía s/n. Puerto de Mazarrón, 30860 Murcia, Spain

[2]Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100 Murcia, Spain

[3]*Dipartimento per l'Innovazione nei Sistemi Biologici Agroalimentari e Forestali,* Università della Tuscia, Italy.

Corresponding author: Alberto Cuesta, Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100 Murcia, Spain. Tel.: +34 868884536; Fax: +34 868883963; E mail: alcuesta@um.es

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Summary

One of the most powerful innate immune responses against virus is mediated by the type I interferon (IFN). In teleost fish, it is known that virus infection triggers the expression of *ifn* and many IFN-stimulated genes but the viral RNA sensors and mediators leading to the IFN production are scarcely known. Thus, we have searched the presence of these genes in gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) and evaluated their expression after infection with viral nervous necrosis virus (VNNV) in the brain, the main viral target tissue, and the gonad, used to transmit the virus vertically. In seabream, a resistant fish species to the VNNV strain used, we found an up-regulation of the genes encoding MDA5, TBK1, IRF3, IFN, Mx and PKR proteins in the brain, which were unaltered in the gonad and could favour the dissemination by gonad fluids or gametes. Strikingly, in European sea bass, a very susceptible species, we identified, in addition, transcripts coding for LGP2, MAVS, TRAF3, TANK and IRF7 and found that all the genes analysed were up-regulated in the gonad but only *mda5*, *lgp2*, *irf3*, *mx* and *pkr* did in the brain. These findings support the notion that the European sea bass brain innate immune response is unable to clear the virus and points to the importance of the gonad immunity to control the dissemination of VNNV to the progenies, an aspect that is worth to investigate in aquatic animals.

Keywords: Nodavirus (VNNV); interferon (IFN) pathway; gonad; gilthead seabream; European sea bass

INTRODUCTION

The innate immune response against virus infections uses different mechanisms such as the interferon (IFN), the complement system or the cytotoxic cells (Ellis, 2001) being the IFN response the most well characterized in fish. Mammalian IFNs have been classified as type I (α, β, ω, ε, and κ), type II (γ), and type III (λ) IFNs (Sadler & Williams, 2008). In fish, apart from the type II, the genome sequencing projects have detected different IFN genes ranging from 1 in fugu (*Takifugu rubripes*) or medaka (*Oryzias latipes*) to 11 genes in Atlantic salmon (*Salmo salar*) belonging to the types I and III (Sun *et al*., 2009; Zou and Secombes, 2011). Evolutionary and phylogenetical studies have demonstrated the problems in the fish *ifn* gene nomenclature. In fact, they share characteristics with the mammalian type I and III IFNs, and act as co-orthologs, being suggested to be renamed as IFNφ (Hamming *et al.*, 2011; Levraud *et al.*, 2007). Fish IFNs can be divided into two groups: 2 cysteine-containing group I and 4 cysteine-containing group II (Zou et al., 2007). In addition, group I *ifn* can be subdivided into subgroup-a and subgroup-d and the group II into subgroup-c and subgroup-b. Group I *ifn* genes are found in all the fish species whilst the group II is only found in the most primitive fish such as salmonids and cyprinids (Sun et al., 2009; Zhang et al., 2012; Zou et al., 2007). Therefore, several names have been proposed for fish IFNs: type I IFNs, virus-induced IFNs, IFNλ, IFNφ or even simply IFNs (Langevin *et al.*, 2013). Although, it is demonstrated that fish virus-induced IFNs are structurally type I IFNs, a consensus about a consistent nomenclature for these cytokines has still to be reached. Apart from the controversies in the IFN nomenclature, all these fish type I IFNs have been shown to be induced by virus infections and mediate a type I IFN response by the use of Jak-Stat (Janus kinase-signal transducer and activator of transcription) pathway. Their activation create in the cells an antiviral state through the induction of many IFN-stimulated genes (ISGs), including genes such as the antiviral molecule myxovirus (influenza) resistance protein (Mx), with a direct antiviral activity (Verrier *et al.*, 2011). Thus, most of the studies in fish use the expression of *mx* genes as an indicator of viral infection and activation of the type I IFN response although the cellular components sensing the viral genomes and leading to the IFN response have already been characterized (Aoki *et al.*, 2013; Zou *et al.*, 2009).

Pathogen-associated molecular patterns (PAMPs) are detected by germline-encoded pattern recognition receptors (PRRs) and among them the most studied are the Toll-like receptors (TLRs), followed by retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-oligomerization domain (NOD)-like receptors (NLRs). In the case of fish viruses, TLR3 and TLR22 are induced by dsRNA viruses (Matsuo *et al.*, 2008), whilst TLR7 and TLR8 are by ssRNA viruses (Crozat & Beutler, 2004), which in both cases induces a type I IFN-mediated response. To date, the

involvement of the RLRs in the induction of the type I IFN response is the best characterized (Hansen *et al.*, 2011). This family has three members: RIG-I (also known as DDX58), MDA5 (Melanoma Differentiation-Associated gene 5 or IFIN1) and LGP-2 (Laboratory of Genetics and Physiology 2 or DHX58). These sensors are up-regulated by viral haemorrhagic septicaemia virus (VHSV), spring viremia of carp virus (SVCV), grass carp reovirus (GCRV), viral nervous necrosis virus (VNNV) or infectious pancreatic necrosis virus (IPNV), as well as by polyinosinic acid (poly I:C; a synthetic analogue of viral dsRNA), leading to an increase in the IFN-mediated antiviral response (Chen *et al.*, 2015; Feng *et al.*, 2011; Rise *et al.*, 2008; Rise *et al.*, 2010; Skjesol *et al.*, 2011; Su *et al.*, 2010; Yang *et al.*, 2011). However, further studies are needed to definitely define their role in the antiviral response and the identification and characterization of their mediators in the molecular pathway leading to the IFN activation.

In all vertebrates, the gonad is considered an immunologically-privileged site, as also occurs with the brain and retina, where the immune response proceeds in a different manner in order to avoid cell damage (Chaves-Pozo *et al.*, 2005; Hedger, 2002), and therefore, it is used by some pathogens to be hidden and scape to the immunological control. VNNV, or nodavirus, a bipartite and positive single-stranded RNA virus, is a known vertical and horizontal transmitted pathogen (Arimoto *et al.*, 1992; Kuo *et al.*, 2012) able to infect more than 50 marine fish species, some of them especially sensitive, as the European sea bass (*Dicentrarchus labrax*), and others only susceptible to some strains, as occurs with the gilthead seabream (*Sparus aurata*) (Castric *et al.*, 2001; Frerichs *et al.*, 1996). Interestingly, though the main target tissues of VNNV are the brain and the retina (Castric *et al.*, 2001; Frerichs *et al.*, 1996), both immune-privileged tissues, as the gonad, the virus has also been detected in the European sea bass liver, spleen and caudal fin (López-Jimena *et al.*, 2012) and more recently we have also found it into, and isolated from, the gonad (Valero *et al.*, 2014). Previous studies have documented that VNNV infection induces the immune response with especial emphasis in the type I IFN response. Thus, expression of *ifn* and/or *mx* genes was greatly up-regulated in the brain or immune-relevant tissues of gilthead seabream, orange-spotted grouper (*Epinephelus coioides*) or Atlantic halibut (*Hippoglossus hippoglossus*) but lightly in the European sea bass (Chaves-Pozo *et al.*, 2012; Chen *et al.*, 2014; López-Muñoz *et al.*, 2012; Overgard *et al.*, 2012; Poisa-Beiro *et al.*, 2008; Scapigliati *et al.*, 2010). In addition, *mda5* and *lgp2* transcription was also up-regulated in the brain of gilthead seabream (Dios *et al.*, 2007) and Atlantic cod (*Gadus morhua*) (Rise *et al.*, 2010) by VNNV infection. Unfortunately, any study has investigated the IFN response into the gonad of VNNV-infected fish taking into consideration that this virus uses the gonad to hide and be transmitted.

Taking in mind the previous information, we aimed in this study to deepen in the characterization of the type I IFN pathway of European sea bass and gilthead seabream, and its involvement upon infection with VNNV, as well as in their respective cell lines, focusing on the gonad, and compared to that found in the brain, the target tissue for VNNV.

RESULTS

Identification of genes involved in the IFN pathway

We have identified most of the known genes involved in the RLR-activation pathway of the IFN (Fig. 1). In gilthead seabream and European sea bass fish species, *ifn* and *mx* genes have already been characterized (Casani *et al.*, 2009; Fernández-Trujillo *et al.*, 2011; Scapigliati *et al.*, 2010). Searching the EST databases, we found partial or full-length sequences of seabream *mda5*, *tbk1*, *irf3* and *pkr* genes as well as European sea bass *mda5*, *lgp2*, *irf3* and *pkr*, which were expanded to *mavs*, *traf3*, *tank* and *irf7* by searching a sea bass gill transcriptome obtained by RNA-seq (Nuñez Ortiz *et al.*, 2014). However, we did not investigate the presence of multiple gene copies or alternative splicing forms. As previously demonstrated (Zou *et al.*, 2009), we also failed to find any *rig1* mRNA sequences in the seabream and sea bass, both belonging to the modern teleosts. The predicted length, homology and e-values obtained from the gene sequences were compared with their zebrafish orthologs (Table 1) resulting in *bona fide* sequences, which was further confirmed by the analysis of the predicted protein domains and its conservation (Supplementary data; Table S1). These domains include: helicase in MDA5 and LGP2, CARD in MAVS, RING and MATH_TRAF3 in TRAF3, TBD in TANK, STKc_TBK1 in sea bass TBK1, IRF-3 in both IRF3 and 7, STKc_EIF2AK2_PKR in seabream PKR and DSRM in sea bass PKR. All these domains were also found and conserved in the respective zebrafish and human orthologs.

Genes of the IFN pathway are constitutively expressed

Before determining the effects of any of the *stimuli* on the levels of expression of the different IFN pathway genes, we determined the constitutive levels of expression of these genes in the brain and gonad of naïve gilthead seabream and European sea bass specimens and cell lines (Fig. 2). In gilthead seabream, all genes were similarly expressed in the brain and gonad whilst their transcription levels in the SAF-1 cells were much lower for *pkr*, *ifn* and *mx*. In European sea bass, all the genes were constitutively expressed with little variations between the tissues and usually lower in the DLB-1 cell line, derived from sea bass brain.

Most of the genes were up-regulated in vitro by poly I:C and VNNV infection

In the gilthead seabream SAF-1 cell line, *mda* and *irf3*, but not *tbk1* transcription levels were similarly induced by poly I:C or VNNV, except in the case of *ifn* transcription levels, which were unaffected by poly I:C and greatly up-regulated by VNNV infection (Fig. 3). However, whilst the *mx* gene expression was greatly induced, the *pkr* transcription was down-regulated by both *stimuli*. In a similar way, both poly I:C and VNNV induced most of the genes related to the IFN-production pathway in the sea bass DLB-1 cell line though polyI:C usually provoked a greater induction (Fig. 3). Interestingly, VNNV failed to induce the RNA sensors *mda5* and *lgp2* transcription, although the downstream genes were significantly up-regulated. Moreover, in sea bass DLB-1 cell line, *tbk1* expression resulted unaltered with both, poly I:C and VNNV, whilst *pkr* was increased only with poly I:C treatment.

Sensors of the viral dsRNA are up-regulated in the gonad of VNNV-infected European sea bass

We evaluated the expression of the two identified RLRs, *mda5* and *lgp2*, which are the sensors for dsRNA, after VNNV infection (Fig. 4). In seabream, *mda5* transcription was increased in the brain but unaffected in the gonad. However, in the sea bass, both *mda5* and *lgp2* were similarly regulated upon VNNV infection in both tissues. Thus, in the brain, they were down-regulated after 1 and 7 days of infection to be later on up-regulated. In contrast, these genes were up-regulated in the gonad after 1 and 7 days of infection and unchanged afterwards.

Adaptor and intermediaries are triggered by VNNV infection in the gonad of European sea bass

In gilthead seabream, we only identified the *tbk1* and *irf3* intermediaries (Fig. 5). Transcription of *tbk1* was unaltered by VNNV infection in any tissue whilst *irf3* gene expression was induced after 7 and 15 days of VNNV infection in the brain and only after 1 day in the gonad. In European sea bass, the RLR adaptor, *mavs*, and most of the IFN-production pathway intermediary genes were identified. As occurred with the receptors, all the studied genes were down-regulated in the brain of sea bass infected with VNNV except the *irf3* gene that was induced after 15 days of infection (Fig. 4). By contrast, in the gonad, all of them (*mavs*, *traf3*, *tank*, *tbk1*, *irf3* and *irf7*) were up-regulated at different time points, mainly after 1 and 7 days of infection.

VNNV greatly induced ifn, mx and pkr gene expression in the European sea bass gonad

Finally, the *ifn* gene was unaltered upon VNNV infection in the gilthead seabream brain and reduced its expression in the European sea bass brain (Fig. 6). On the other hand, in the gonad, the *ifn* transcription was decreased in seabream after 15 days of infection but induced in sea bass at days 1 and 7. After IFN production, we evaluated the transcription of two IFN-stimulated genes, which

175 are responsible of the antiviral response, in our case *mx* and *pkr*. Thus, in seabream, both genes were
176 up-regulated upon VNNV infection in the brain, increasing its levels along the infection, but
177 unaltered in the gonad (Fig. 6). By contrast, sea bass brain mRNA levels of *mx* were greatly
178 increased after 1 day of infection and decreased thereafter at day 7 whilst the *pkr* was only induced
179 after 15 days of infection (Fig. 6). In the gonad, however, *mx* was greatly induced after 7 and 15 days
180 of infection but undetected at day 1. Nevertheless, *pkr* transcription was always induced being the
181 highest levels reached at day 1 and decreasing thereafter.

182

183 **DISCUSSION**

184 Gilthead seabream and European sea bass are the most important fish species in the
185 Mediterranean aquaculture. So far, single *ifn* genes, belonging to the type I IFN, have been
186 documented and partially characterized together to the IFN-induced *mx* gene (Casani *et al.*, 2009;
187 Fernández-Trujillo *et al.*, 2011; Scapigliati *et al.*, 2010). Focusing on VNNV, the two viral genes,
188 coding for the capsid and RNA-dependent RNA polymerase, were found them at very low levels in
189 the brain of seabream specimens and increased up to 10^7-fold in the brain of sea bass (Chaves-Pozo
190 et al., 2012). Strikingly, it has been recognized that VNNV infections induce a great type I IFN
191 response in the main target tissue, the brain, and that this activation might be responsible for the viral
192 clearance in the resistant fish species gilthead seabream whilst low activity is observed in those
193 susceptible species such as European sea bass (Chaves-Pozo *et al.*, 2012; Chen *et al.*, 2014; López-
194 Muñoz *et al.*, 2012; Overgard *et al.*, 2012; Poisa-Beiro *et al.*, 2008; Scapigliati *et al.*, 2010).
195 However, very little is known about the molecular mechanisms leading to the type I IFN activation
196 in fish induced by virus, and in particular by VNNV (Dios *et al.*, 2007; Rise *et al.*, 2010). Moreover,
197 none of these studies have looked at the gonad immune response on these species, an issue that it is
198 highlighted taking into account that this tissue is used to vertically transmit VNNV to the progeny
199 (Arimoto *et al.*, 1992; Kuo *et al.*, 2012). Concretely, though we have failed to detect any viral gene
200 expression by conventional and real-time PCR, we have already shown that VNNV is able to
201 replicate into the gonad of gilthead seabream and European sea bass by *in situ* PCR,
202 immunohistochemistry and viral recovery using cell culture (Valero *et al.*, 2014). In addition, and
203 most strikingly, the activity of antimicrobial peptides, and its transcription, was greatly up-regulated
204 in the gonad of VNNV-infected sea bass specimens but failed to do so in the sea bass brain and in the
205 gonad of seabream specimens (Valero *et al.*, 2015). These data point to the importance of the gonad
206 immunity in VNNV establishment and dissemination and prompted us to carry out this study.

We have searched ESTs databases of gilthead seabream and European sea bass as well as European sea bass gill transcriptome to search for RLR genes and mediators leading to IFN production. Firstly, we found some RNA sensors like *mda5* sequences in both fish species and *lgp2* in only sea bass but failed to detect any *rig1* mRNA (Fig. 1). In a similar way, *mda5* and *lgp2* genes have been identified in all teleost fish studied so far though the presence of *rig1* gene is limited to the ancient and never identified in the modern fish (class *Acanthopterygii*) (Aoki *et al.*, 2013), in which our fish species are included. Our data showed that the expression levels of *mda5* was up-regulated in the SAF-1 cell line, which supports VNNV replication (Bandín *et al.*, 2006), in a similar way to the zebrafish ZF-4 cell line, which also supports VNNV replication, in which *rig1*, *mda5* and *lgp2* transcription was up-regulated by VNNV infection (Chen *et al.*, 2015). However, neither *mda5* or *lgp2* genes were altered in the newly obtained sea bass DLB-1 cells in contrast to what happens with poly I:C stimulation. This could indicate that VNNV is not able to replicate into sea bass DLB-1 cells, although this needs to be further confirmed. Moreover, up-regulation of the transcription of *mda5* and *lgp2* after VNNV infection *in vivo* suggests that their production is induced upon viral infection and that they may recognize viral RNA and induce the IFN response. The induction is of particular importance in seabream brain and in sea bass gonad indicating that these tissues would exert a high antiviral response. Similar up-regulations have been already documented in the brain of sea bass or Atlantic halibut exposed to VNNV (Dios *et al.*, 2007; Rise *et al.*, 2010) and support our data. Moreover, these sensors are also up-regulated by several fish RNA virus or poly I:C in several tissues of fish such as spleen, head-kidney, liver or intestine, as well as in some fish cell lines, leading to an increase in the type I IFN-mediated antiviral response (Feng *et al.*, 2011; Rise *et al.*, 2008; Rise *et al.*, 2010; Skjesol *et al.*, 2011; Su *et al.*, 2010; Yang *et al.*, 2011). Moreover, fish *rig1* and *mda5* transient overexpression lead to the induction of the *ifn* expression and conferred an antiviral state (Biacchesi *et al.*, 2009; Sun *et al.*, 2011). Very recently, in addition, *rig1* knock-down in ZF-4 cells has demonstrated the importance of the group II of type I IFN pathway in VNNV infections (Chen *et al.*, 2015). However, *lgp2* overexpression can produce both inducing and inhibitory effects on the *ifn* expression as evidenced in fish and mammals (Komuro & Horvath, 2006; Ohtani *et al.*, 2012; Sun *et al.*, 2011), probably due to the lack of the caspase activation and recruitment domain (CARD), which is only present in RIG-I and MDA5 proteins.

We also investigated the presence and regulation of genes between the RLRs and IFN (Fig. 1). Thus, we looked for and found in the gilthead seabream ESTs databases sequences two intermediates molecules; *tbk1* and *irf3* transcripts, and in the European sea bass we successfully obtained sequences for most of the molecules involved in the INF-induced pathway: *mavs*, *traf3*,

tank, *tbk1*, *irf3* and *irf7* mRNA. Though most of them are only partial sequences the analysis of the predicted proteins resulted in *bona fide* orthologs to the expected proteins. Their expression in naïve conditions and upon VNNV infection in brain and gonad correlated with the expression of *ifn* and two IFN-stimulated genes: *mx* and *pkr*. Regarding these genes, our results showed that VNNV was able to increase the expression of genes related to the RLR adaptor, *mavs*, and intermediaries of the pathway leading to the IFN production. Strikingly, these genes were usually down-regulated in the brain of sea bass specimens infected with VNNV but up-regulated in the gonad. This fact would suggest a high IFN or antiviral response in the sea bass gonad and very low in the brain, which could explain the low resistance of this fish species but this needs to be confirmed at functional level. These results are in agreement with other studies in fish showing the up-regulation of most of these genes after virus infection in several tissues or their antiviral function after cell lines over-expression (Biacchesi *et al.*, 2009; Chen *et al.*, 2015; Feng *et al.*, 2011; Rise *et al.*, 2008; Rise *et al.*, 2010; Skjesol *et al.*, 2011; Su *et al.*, 2010; Sun *et al.*, 2011; Xiang *et al.*, 2011; Yang *et al.*, 2011) and support the fact that the sequences identified in our study are mediating in the IFN activation cascade. In the case of *tbk1*, which is also activated by the TLR response, it is only up-regulated in sea bass specimens infected with VNNV. However, fish *tbk1* has been shown to be activated by virus, poly I:C, peptidoglycan and/or lipopolysaccharide indicating that this molecule can be activated by both viral and bacterial pathogens (Chi *et al.*, 2011; Feng *et al.*, 2011; Feng *et al.*, 2014; Zhang *et al.*, 2014). Moreover, some data point to the activation of *tbk1* and the antiviral response without the major involvement of IRF3/7 pointing to the existence of other activation pathways in fish (Feng et al., 2014). Now, our data showed that in the case of gilthead seabream which is able to clear the VNNV infection (Chaves-Pozo *et al.*, 2012), *tbk1* expression is not up-regulated suggesting that this molecule is not essential to gilthead seabream anti-viral immune response.

Finally, this cascade leads to the activation of the IFN response (Fig. 1). Our data showed that *ifn* transcription in gilthead seabream was not achieved though the down-stream activation of IFN-stimulated genes such as *mx* and *pkr* that were mainly observed in the brain of VNNV-infected specimens. This could be explained by the different induction times, since *ifn* expression is usually very fast and last for short period, or to the presence of different *ifn* forms and splicing variants, which is unknown so far and deserves further work. By contrast, in the European sea bass, inhibition of the brain expression of *ifn* gene, as most of those genes involved in the induction cascade, was concomitant with an increase in the transcription of *mx* and *pkr*. All this data pointed to the existence of other activation pathways in fish as previously suggested (Feng et al., 2014) and demonstrated in ZF-4 cells in which the involvement of the TLR activation pathway is evidenced after VNNV

273 infection (Chen *et al.*, 2015). In addition, *pkr* is designed as an IFN-stimulated gene but it is able to
274 directly recognize and bind to viral RNA and therefore might be considered as another PRR. This
275 could be supported by the finding that ZF-4 cells knocked down in *rig1* and infected with VNNV
276 showed an up-regulated *pkr* expression (Chen *et al.*, 2015). Interestingly, in the gonad of VNNV-
277 infected sea bass specimens, *ifn*, *mx* and *pkr* genes were also up-regulated as occurred with the
278 sensors and intermediary genes. In previous studies, the induction of the IFN pathway after viral
279 infection has been evaluated in several immune-relevant tissues (Chi *et al.*, 2011; Feng *et al.*, 2011;
280 Feng *et al.*, 2014), but never included the fish gonad. This is important since it is known that gonad
281 immunity is tissue-specifically regulated in fish (Chaves-Pozo *et al.*, 2005) and used by pathogens
282 for its dissemination (Arimoto *et al.*, 1992; Kuo *et al.*, 2012). The up-regulation of the antiviral
283 response in the gonad of European sea bass specimens surviving to the VNNV infection could be a
284 mechanism in which fight the pathogen is more important than maintain the functionality of the
285 gonad for reproductive purposes. However, in the gilthead seabream, specimens which overcome the
286 infection, the tight regulation of the gonadal immune response could avoid germ cell damage but at
287 the same time allow the transmission of the virus through the gonad fluids and gametes. This
288 hypothesis is supported by the fact that, when other immune molecules such as antimicrobial
289 peptides, are studied their expression pattern in the brain and gonad of VNNV-infected sea bass are
290 similar (Valero *et al.*, 2015). However, the antiviral immune response in the reproductive organs
291 deserved further investigation since in immature rainbow trout (*Oncorhynchus mykiss*) females,
292 VHSV infection provoked an up-regulation of the type I IFN genes (*ifn1*, *ifn2*, *ifn3/4*, *mx1*, *mx2* and
293 *mx3*) in the ovary (Chaves-Pozo *et al.*, 2010). In addition, recombinant IFN1 and IFN2 were able to
294 induce the expression of *mx* genes and confer antiviral activity against VHSV *in vitro*, being the *mx3*
295 which showed the highest up-regulation (Chaves-Pozo *et al.*, 2010). This points to the importance of
296 the gonad IFN response to control the dissemination of viral pathogens in fish, an aspect that has
297 been clearly unconsidered in the past.

298 In conclusion, this study represents one of the most complete characterizations of the genes
299 leading to the IFN response after viral infection by RLRs in fish. Thus, we have identified several
300 molecules of gilthead seabream and European sea bass involve in the activation cascade of the
301 interferon including viral RNA receptors (*mda5* and *lgp2*), the RLR adaptor (*mavs*) and
302 intermediaries (*traf3*, *tank*, *tbk1*, *irf3* and *irf7*) for the first time. We also reported their simultaneous
303 regulation upon VNNV infection. Thus, in seabream, we found that *mda5*, *irf3*, *mx* and *pkr* genes
304 were up-regulated in the brain but not in the gonad. However, in the susceptible European sea bass,
305 the expression of most of the genes were down-regulated in the brain but significantly up-regulated

306 in the gonad what resulted in an enhanced transcription of *ifn*, *mx* and *pkr* genes in this tisue. This is
307 the first time since a study covered a wide view of the fish IFN pathway after viral infection and has
308 also included the gonad as an important tissue where the virus might be hidden and transmitted to the
309 progeny.

310 **METHODS**

311 ***Animals* and cell lines.** Adult specimens of the marine teleost gilthead seabream (*Sparus aurata*)
312 and European sea bass (*Dicentrarchus labrax*) (125 ± 25 and 305 ± 77 g body weight, respectively)
313 were bred at the *Centro Oceanográfico de Murcia* (IEO) with natural conditions of photoperiod,
314 temperature, salinity and aeration and translated to the University of Murcia aquaria. Fish were kept
315 in 450-500 L running seawater (28‰ salinity) aquaria at 24 ± 2ºC and with a 12 h light:12 h dark
316 photoperiod and fed daily with 1 g per fish of a commercial pellet diet (Skretting). Animals were
317 acclimatized for 15 days prior to the experiments. All animal studies were carried out in accordance
318 with the Guidelines of the European Union Council (2010/63/UE), the Bioethical Committee of the
319 University of Murcia (Spain) and the *Instituto Español de Oceanografía* (Spain) for the use of
320 laboratory animals.

321 Cell lines were cultured at 25ºC in 25 cm^2 plastic tissue culture flasks (Nunc) and maintained
322 at exponential growth. The established striped snakehead SSN-1 (Frerichs *et al.*, 1996) and seabream
323 SAF-1 (Béjar *et al.*, 2005) cell lines were cultured using Leibovitz's L15-medium (Life
324 Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 2 mM L-
325 glutamine (Life Technologies), 100 i.u. ml^{-1} penicillin (Life Technologies) and 100 µg ml^{-1}
326 streptomycin (Life Technologies) whilst a new cell line derived from the European sea bass brain
327 (DLB-1) obtained in our laboratory was cultured using Eagle's Minimal Essential Medium (EMEM;
328 Life Technologies) supplemented with 15% FBS, glutamine and antibiotics as above.

329 **VNNV stocks.** VNNV (strain 411/96, genotype RGNNV) were propagated in the SSN-1 cell line
330 which is persistently infected with a snakehead retrovirus (SnRV) (Frerichs *et al.*, 1996). Cells were
331 inoculated with VNNV and incubated at 25ºC until the cytopathic effect was extensive. Supernatants
332 were harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates
333 before used in the experiments (Reed & Muench, 1938).

334 **Gene search and bioinformatic analysis.** According to the literature (Sun *et al.*, 2011; Takeuchi &
335 Akira, 2008; Zhang *et al.*, 2014), virally activated RLRs (MAD5, LGP2 or RIG-I) initiate a
336 molecular pathway leading to the expression of *ifn* and IFN-induced genes creating the cellular
337 antiviral state. Thus, these receptors interact with the RLR adaptor protein, MAVS (or the IFN-β
338 promoter stimulator-1 IPS-1), then it associates with tumor necrosis factor (TNF) receptor-associated

339 factor 3 (TRAF3), which recruits and facilitates the interaction between, but not exclusively, TRAF
340 family member-associated NF-kB activator (TANK) and TANK-binding kinase 1 (TBK1), also
341 activated by TLR3, and therefore the TLR and RLR IFN-activation pathways by viral RNA are
342 shared from this point. TBK1, in turns, phosphorylates and activates IFN regulatory factors (IRF)-3
343 and -7. These IRF3 and 7 are then translocated to the nucleus where bind to the IFN-stimulated
344 response elements (ISRE) and activate the expression of *ifn* and IFN-stimulated genes, including the
345 Mx and PKR (dsRNA-dependent protein kinase receptor) coding genes.
346 Therefore, in this work, the corresponding coding sequences for zebrafish proteins were
347 selected and launched using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) within the
348 expressed sequence tags (ESTs) databases from gilthead seabream and European sea bass as well as
349 within the European sea bass gill transcriptome (Nuñez Ortiz *et al.*, 2014). Thus, deduced protein
350 sequences, from the full or partial gene sequences were obtained and analyzed for similarity with
351 known ortholog sequences and domain conservation using the BLAST program (Altschul *et al.*,
352 1990) within the ExPASy Molecular Biology server (http://us.expasy.org). Phylogenetic and
353 molecular evolutionary analyses were conducted using MEGA version 6 (Tamura *et al.*, 2013) to
354 confirm that they are expected *bona fide* sequences. The sequences found and studied, related to the
355 IFN pathway activation by RLRs, are described in this work (Fig. 1).
356 **In vitro infections**. Duplicate cultures of SAF-1 and DLB-1 cells were incubated for 24 h with
357 culture medium alone (controls) or containing 50 μg ml^{-1} polyinosinic acid (pI:C) or 10^6 $TCID_{50}$ ml^{-1}
358 VNNV. After treatment, monolayers were carefully washed with PBS and stored in TRIzol Reagent
359 (Life Technologies) at -80ºC for latter isolation of RNA.

360 **In vivo infections with VNNV.** Thirty specimens of gilthead seabream or European sea bass were
361 randomly divided into two tanks. Each group received a single intramuscular injection of 100 μl of
362 SSN-1 culture medium (mock-infected) or culture medium containing 10^6 VNNV $TCID_{50}$ $fish^{-1}$ since
363 this route of infection has been proven as the most effective (Aranguren *et al.*, 2002). Fish were
364 sampled 1, 7 and 15 days after the viral injection and fragments of brain and gonad tissues were
365 stored in TRIzol Reagent at -80ºC for latter isolation of RNA.
366 **Analysis of gene expression by real-time PCR.** We studied the transcription of selected genes in
367 brain and gonad from naïve fish, SAF-1 and DLB-1 cell lines, as well as after *in vitro* treatments
368 with pI:C or VNNV and after *in vivo* infection with VNNV. Total RNA was isolated from TRIzol
369 Reagent frozen samples following the manufacturer's instructions. One μg of total RNA was treated
370 with DNAse I to remove genomic DNA and the first strand of cDNA synthesized by reverse

transcription using the SuperScript^{TM} III Reverse Transcriptase (Invitrogen) with an oligo-dT_{12-18} primer (Invitrogen) followed by RNAse H (Invitrogen) treatment.

Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC and 15s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1α (*ef1a*) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct. Gene names follow the accepted nomenclature for zebrafish (https://wiki.zfin.org). The primers used were designed using the Oligo Perfect software tool (Invitrogen) and are shown in Table 2. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. Amplified products from positive samples were run in 2% agarose gels and sequenced. After these verifications, all amplifications were performed in duplicate cDNAs and repeated once to confirm the results. Negative controls with no template were always included in the reactions.

Statistical analysis. Data in figures are represented as mean ± SEM (n = 4-6 individuals in the *in vivo* experiment and n = 2 independent *in vitro* experiments). Statistical differences between control and treated groups were analyzed by one-way analysis of variance (ANOVA; $p \leq 0.05$) using the SPSS 20 software.

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555 **Table 1.** Identification of the selected genes in the expressed sequence tags (ESTs) databases and
556 European sea bass gill transcriptome and their relation with the zebrafish orthologs.

Predicted protein	**Fish species**	**Gene accession number**	**Protein length**	**% protein homology[a]**	**e-value[b]**
MDA5	Seabream Sea bass Zebrafish	HS988207 AM986362 XP_694124	289 206 997*	71 72	1e-123 1e-91
LGP2	Sea bass Zebrafish	AM984225 NP_001244086	297 679*	71	2e-115
MAVS/IPS-1	Sea bass Zebrafish	KP861888 XP_005156619	586* 585*	42	3e-18
TRAF3	Sea bass Zebrafish	KP861887 NP_001003513	595* 573*	74	0.0
TANK	Sea bass Zebrafish	KP861886 NP_001070068	242 348*	44	6e-42
TBK1	Seabream Sea bass Zebrafish	HS988213 FM013306 NP_001038213	301 220 727*	77 95	5e-154 3e-33
IRF3	Seabream Sea bass Zebrafish	AM956899 CBN81356 NP_001137376	201 465* 426*	44 41	3e-47 2e-87
IRF7	Sea bass Zebrafish	KP861885 NP_956971	433* 423*	51	4e-135
PKR	Seabream Sea bass Zebrafish	HS988732 FM008342 CAM07151	306 304 682*	52 41	3e-88 1e-41

557 Percentage of homology ([a]) and e-value ([b]) of the predicted proteins respect to the zebrafish ortholog.
558 Asterisk denotes the sequences with predicted full length.

559

560 **Table 2.** Primers used for analysis of gene expression by real-time PCR.

Gene name	Gene abbreviation	Fish specie	Acc. numbers	Sequence (5'-3')
Melanoma differentiation-associated 5 protein	*mda5*	Seabream	HS988207	CATCGAGATCATCGAGGACA
				CCAGATGTCGCTCTTGAAGG
		Sea bass	AM986362	AATTCGGCAATGGTGAAGTC
				TCATTGGTCACAAGGCCATA
Laboratory of genetics and physiology 2 protein	*lgp2*	Sea bass	AM984225	TGATGGCAGTCAGTGGAGAG
				TGAGAGCTCAACGTGTTTGG
Mitochondrial antiviral-signaling protein	*mavs*	Sea bass	KP861888	GCACAAGCTCAAAGCATCAA
				TCACTGGAGGGGGTGTTTAC
TNF receptor-associated factor 3	*traf3*	Sea bass	KP861887	CGATTAGCCGACATGGATCT
				TGCTTCCTGTTTCCGTCTCT
TRAF family member-associated nuclear factor-kappa-B activator	*tank*	Sea bass	KP861886	GCGGACAGCGAATATGACTT
				GCAATGTGGAGGGGACACTA
TANK-binding kinase 1	*tbk1*	Seabream	HS988213	AGGAACAGCTGCCTCAGAAG
				CAGCTTCTTCATCCCCAGAG
		Sea bass	FM013306	ACAAGGTCCTGGTGATGGAG
				CGTCCTCAGGAAGTCCGTAA
Interferon regulatory factor 3	*irf3*	Seabream	AM956899	TCAGAATGCCCCAAGAGATT
				AGAGTCTCCGCCTTCAGATG
		Sea bass	CBN81356	AGAGGTGAGTGGCAATGGTC
				GAGCAGTTTGAAGCCTTTGG
Interferon regulatory factor 7	*irf7*	Sea bass	KP861885	ATTCACCAACCGCATCCTTA
				GCCTCCAGGCATAGATACCA
dsRNA-dependent protein kinase receptor	*pkr*	Seabream	HS988732	TCCTTTGGAACCTCCCTACC
				TCGAGGGGGAAATGTTGTAA
		Sea bass	FM008342	AGGGTCAGAGCATCAAGGAA
				GACACCTTGCTGTCCCAGTC
Type I Interferon	*ifn*	Seabream	FM882244	ATGGGAGGAGAACACAGTGG
				GGCTGGACAGTCTCTGGAAG
		Sea bass	AM765847	GGCTCTACTGGATACGATGGC
				CTCCCATGATGCAGAGCTGTG
Myxovirus (influenza) resistance proteins	*mx*	Seabream	FJ490556, FJ490555, FJ652200	AAGAGGAGGACGAGGAGGAG
				TTCAGGTGCAGCATCAACTC
		Sea bass	AM228977, HQ237501, AY424961	GAAGAAGGGCTACATGATCGTC
				CCGTCATTGTAGAGAGTGTGGA
Elongation factor 1 alpha	*ef1a*	Seabream	AF184170	CTGTCAAGGAAATCCGTCGT
				TGACCTGAGCGTTGAAGTTG
		Sea bass	FM019753	CGTTGGCTTCAACATCAAGA
				GAAGTTGTCTGCTCCCTTGG

561

562

563 **Figure legends**

564 **Fig. 1.** RLR-activation of the IFN response in gilthead seabream and European sea bass. RLR
565 [retinoic-acid-inducible gene I (RIG-I)-like receptors), MDA5 (Melanoma Differentiation-
566 Associated 5], LGP2 (Laboratory of Genetics and Physiology 2), MAVS (Mitochondrial antiviral-
567 signaling protein), TRAF3, [tumor necrosis factor (TNF) receptor-associated factor 3], TANK
568 (TRAF family member-associated NF-kB activator), TBK1 (TANK-binding kinase 1), IRF3 or 7
569 [interferon (IFN) regulatory factor 3 or 7], Mx [myxovirus (influenza) resistance proteins], PKR
570 (dsRNA-dependent protein kinase receptor), ISRE (IFN-stimulated response elements), ISG (IFN-
571 stimulated genes). This figure contains the molecules found and analysed in this study and is inspired
572 in the literature (Aoki *et al.*, 2013; Hansen *et al.*, 2011; Takeuchi & Akira, 2008; Verrier *et al.*, 2011;
573 Zhang *et al.*, 2014).

574 **Fig. 2.** Expression of genes related to the IFN-induced response pathway in naïve gilthead seabream
575 and European sea bass. The constitutive mRNA level of genes was studied by real-time PCR from
576 naïve brain, gonad or cell lines. Data represent mean relative expression to the expression of
577 endogenous control *ef1a* gene ± SEM of six specimen tissues or two cell cultures.

578 **Fig. 3.** Poly I:C and VNNV treatment up-regulates most of the IFN-production pathway genes
579 (abbreviated as in Figure 1) in SAF-1 and DLB-1 cell lines derived from gilthead seabream and
580 European sea bass, respectively. Results are expressed as the mean ± SEM (two independent
581 experiments) of mRNA fold increase respect to control samples. Significant differences (ANOVA,
582 $P \leq 0.05$) with the controls are denoted by an asterisk.

583 **Fig. 4.** *In vivo* VNNV infection modifies the expression of the sensors *mda5* and *lgp2* in the brain
584 and/or gonad. Gene expression was studied by real-time PCR after 1, 7 and 15 days of infection (10^6
585 $TCID_{50}$ per fish) in the brain and gonad tissues. Results are expressed as the mean ± SEM (n=4–6) of
586 mRNA fold increase respect to control samples. Significant differences (ANOVA, $P \leq 0.05$) with the
587 controls at each sampling time are denoted by an asterisk.

588 **Fig. 5.** *In vivo* VNNV infection modifies the expression of *tbk1* and *irf3* genes in gilthead seabream
589 and *mavs*, *traf3*, *tank*, *tbk1*, *irf3* and *7* genes in European sea bass. Gene expression was studied by
590 real-time PCR after 1, 7 and 15 days of infection (10^6 $TCID_{50}$ per fish) in the brain and gonad tissues.
591 Results are expressed as the mean ± SEM (n=4–6) of mRNA fold increase respect to control
592 samples. Significant differences (ANOVA, $P \leq 0.05$) with the controls at each sampling time are
593 denoted by an asterisk.

594 **Fig. 6.** *ifn*, *mx* and *pkr* gene expressions are regulated upon VNNV infection in gilthead seabream

595 and European sea bass specimens. Gene expression was studied by real-time PCR after 1, 7 and 15
596 days of infection (10^6 $TCID_{50}$ per fish) in the brain and gonad tissues. Results are expressed as the
597 mean ± SEM (n=4–6) of mRNA fold increase respect to control samples. Significant differences
598 (ANOVA, $P \leq 0.05$) with the controls at each sampling time are denoted by an asterisk. ND, not
599 detected

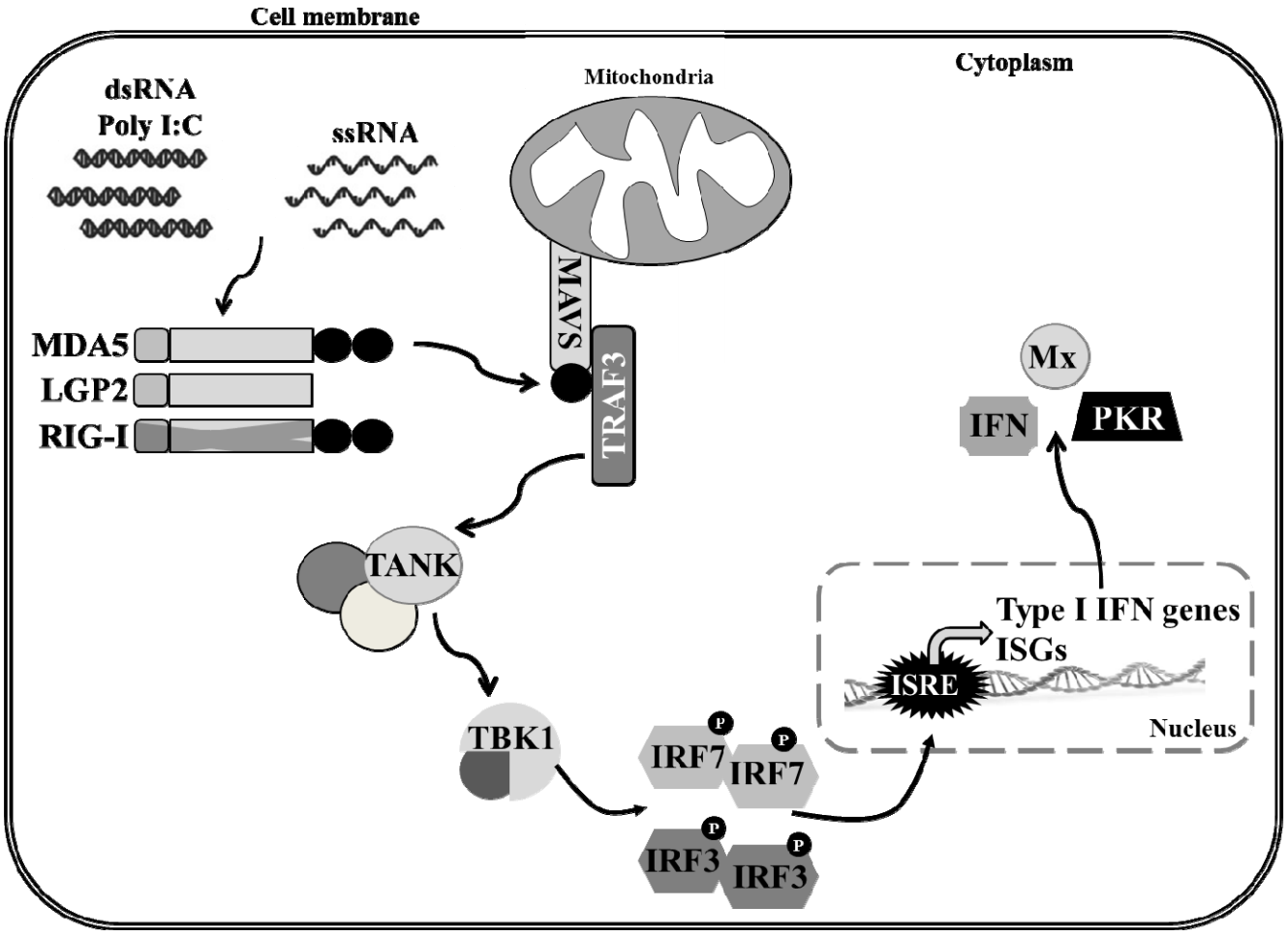
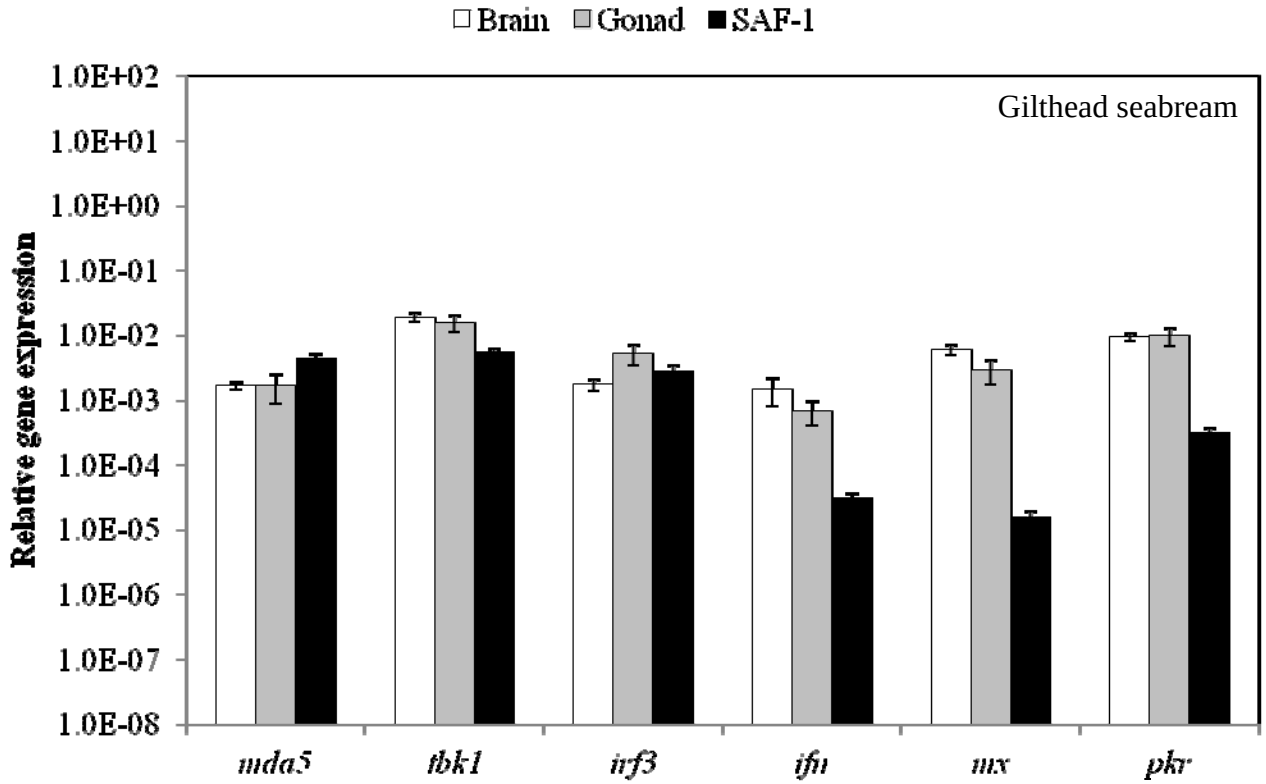

Cell membrane
Cytoplasm
Mitochondria
dsRNA
Poly I:C
ssRNA
MDA5
LGP2
RIG-I
MAVS
TRAF3
TANK
TBK1
IRF7
IRF7
IRF3
IRF3
P
Mx
IFN
PKR
Type I IFN genes
ISGs
ISRE
Nucleus

Figure 1


Brain
Gonad
SAF-1
Gilthead seabream
Relative gene expression
1.0E+02
1.0E+01
1.0E+00
1.0E-01
1.0E-02
1.0E-03
1.0E-04
1.0E-05
1.0E-06
1.0E-07
1.0E-08
mda5
tbk1
irf3
ifn
mx
pkr

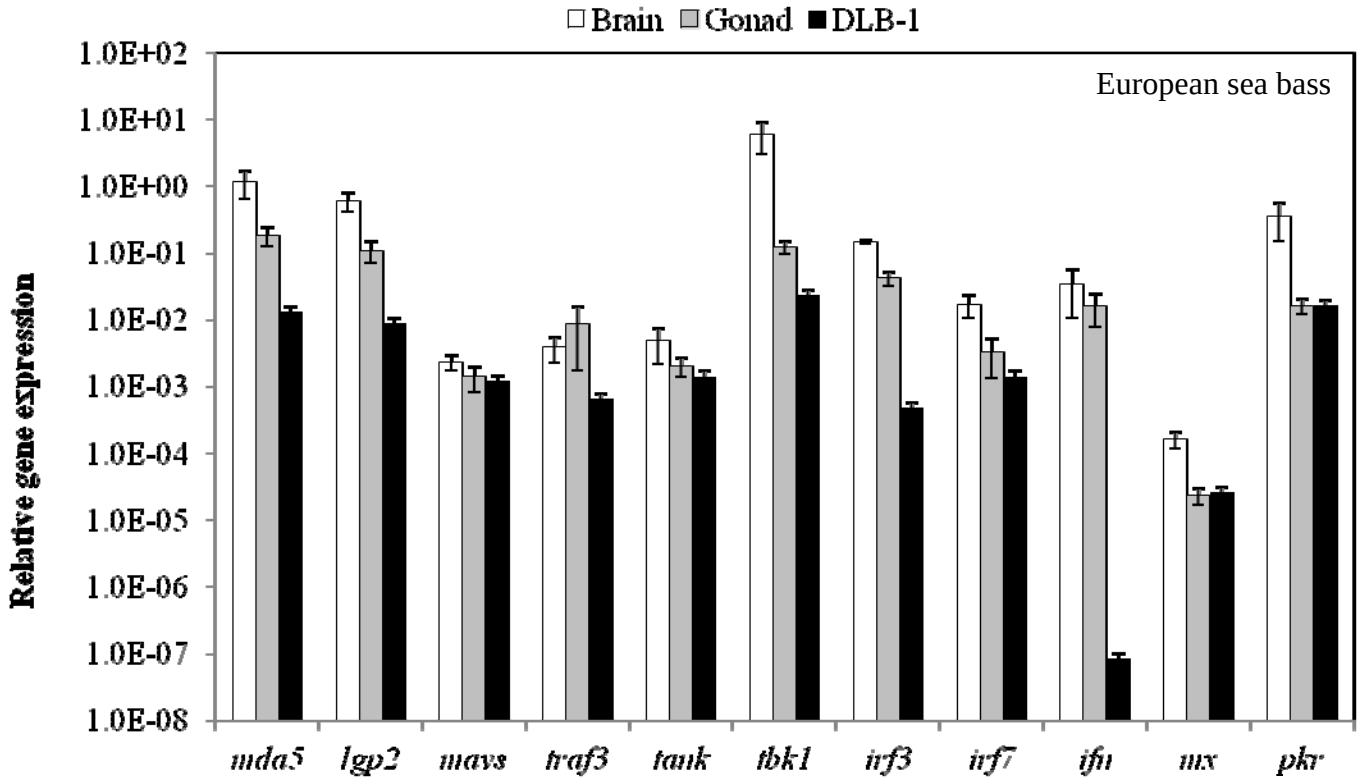

Brain
Gonad
DLB-1
European sea bass
Relative gene expression
1.0E+02
1.0E+01
1.0E+00
1.0E-01
1.0E-02
1.0E-03
1.0E-04
1.0E-05
1.0E-06
1.0E-07
1.0E-08
mda5
lgp2
mavs
traf3
tank
tbk1
irf3
irf7
ifn
mx
pkr

Figure 2

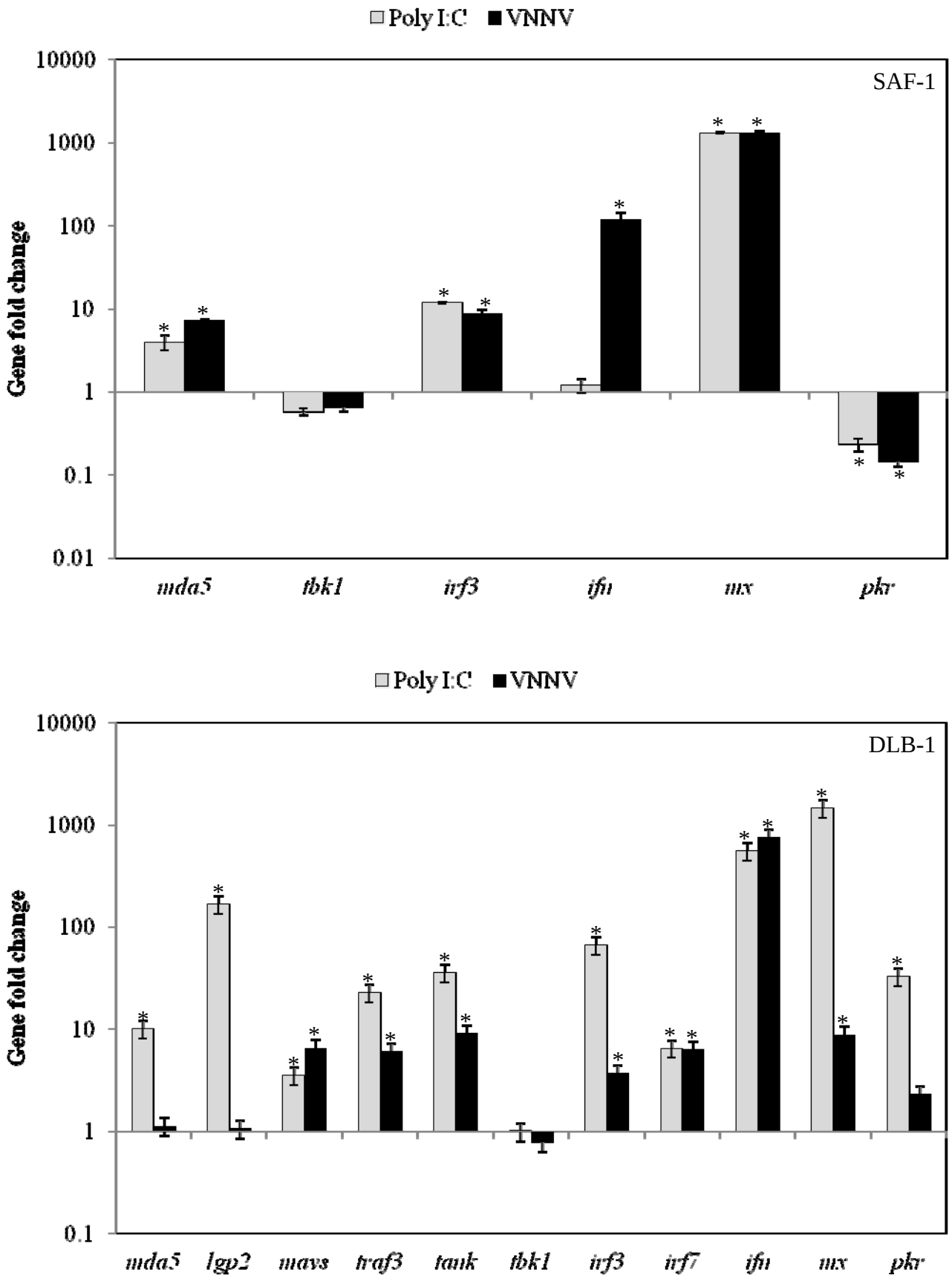
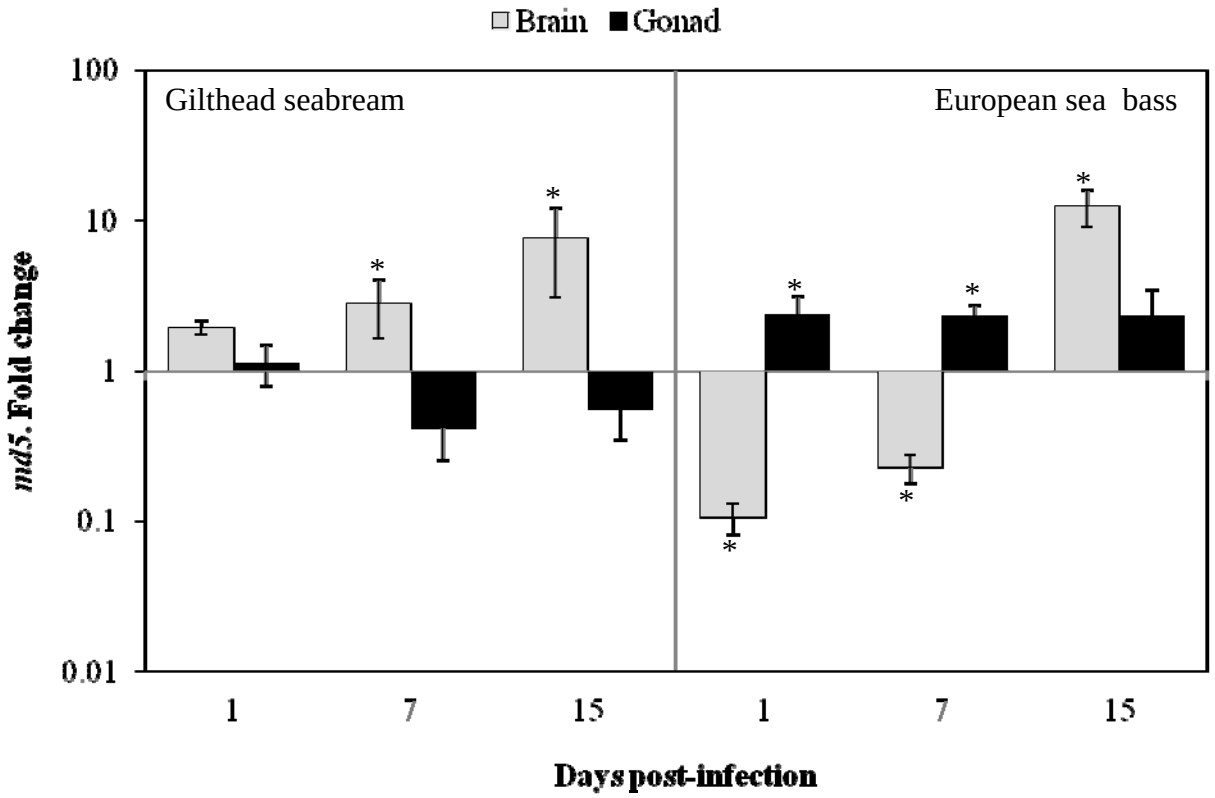

Poly I:C
VNNV
SAF-1
Gene fold change
10000
1000
100
10
1
0.1
0.01
mda5
tbk1
irf3
ifn
mx
pkr
Poly I:C
VNNV
DLB-1
Gene fold change
10000
1000
100
10
1
0.1
mda5
lgp2
mavs
traf3
tank
tbk1
irf3
irf7
ifn
mx
pkr

Figure 3


Brain
Gonad
Gilthead seabream
European sea bass
md5. Fold change
100
10
1
0.1
0.01
1
7
15
1
7
15
Days post-infection

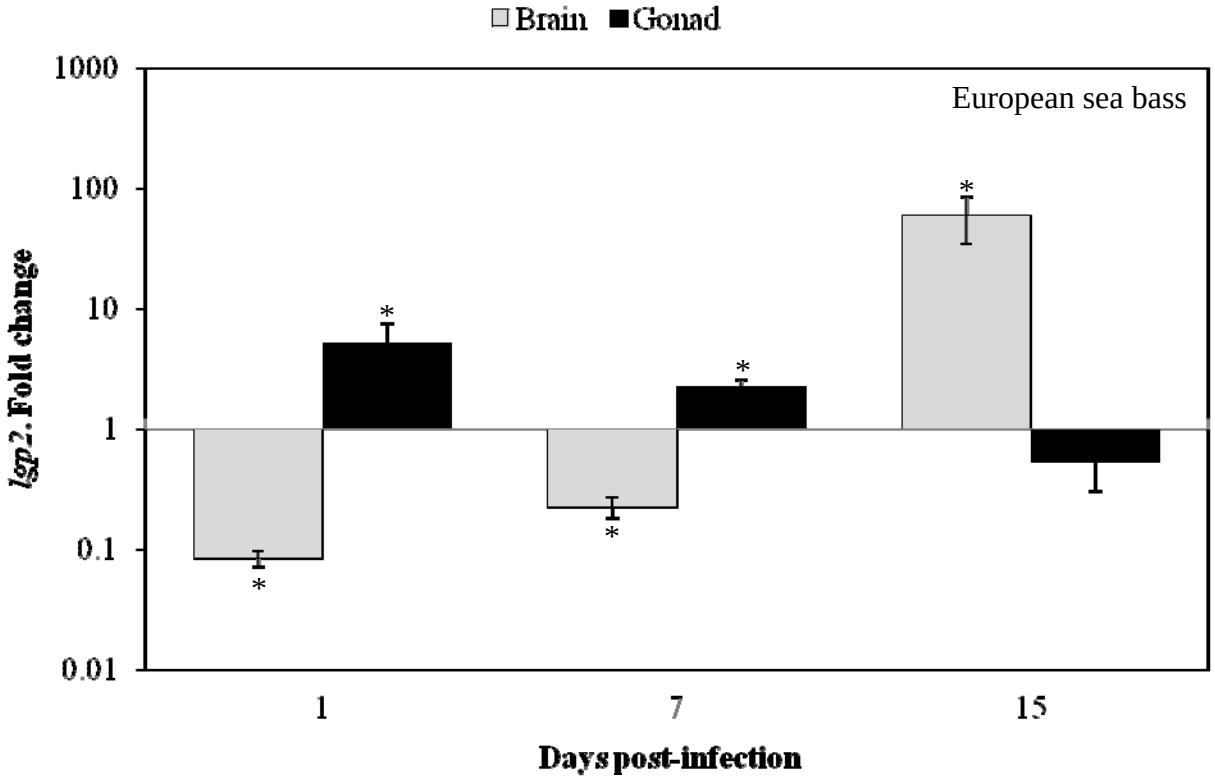

Brain
Gonad
European sea bass
lgp2. Fold change
1000
100
10
1
0.1
0.01
1
7
15
Days post-infection

Figure 4

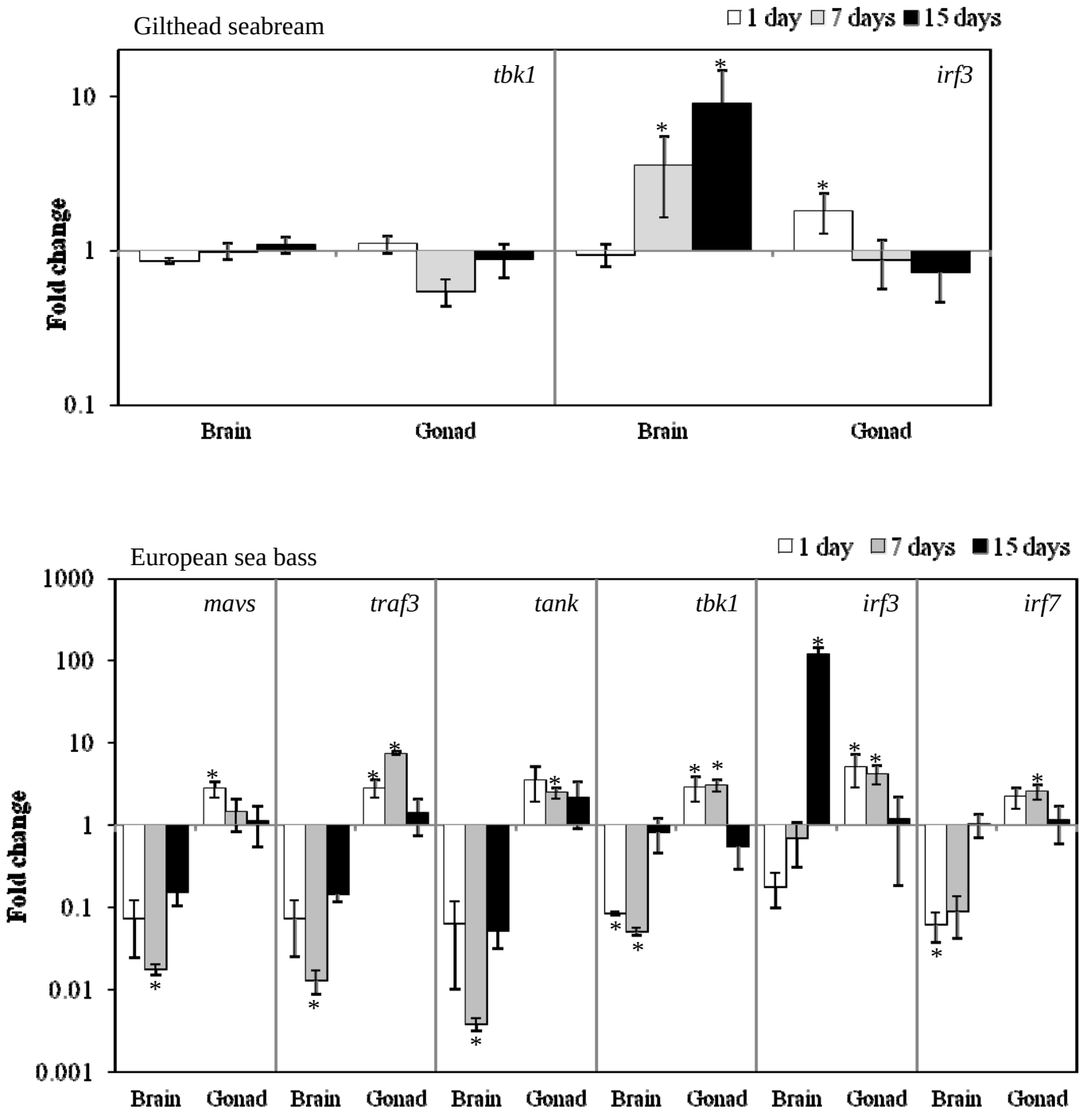

Gilthead seabream
1 day
7 days
15 days
tbk1
irf3
Fold change
10
1
0.1
Brain
Gonad
Brain
Gonad
European sea bass
1 day
7 days
15 days
mavs
traf3
tank
tbk1
irf3
irf7
Fold change
1000
100
10
1
0.1
0.01
0.001
Brain
Gonad
Brain
Gonad
Brain
Gonad
Brain
Gonad
Brain
Gonad
Brain
Gonad

Figure 5

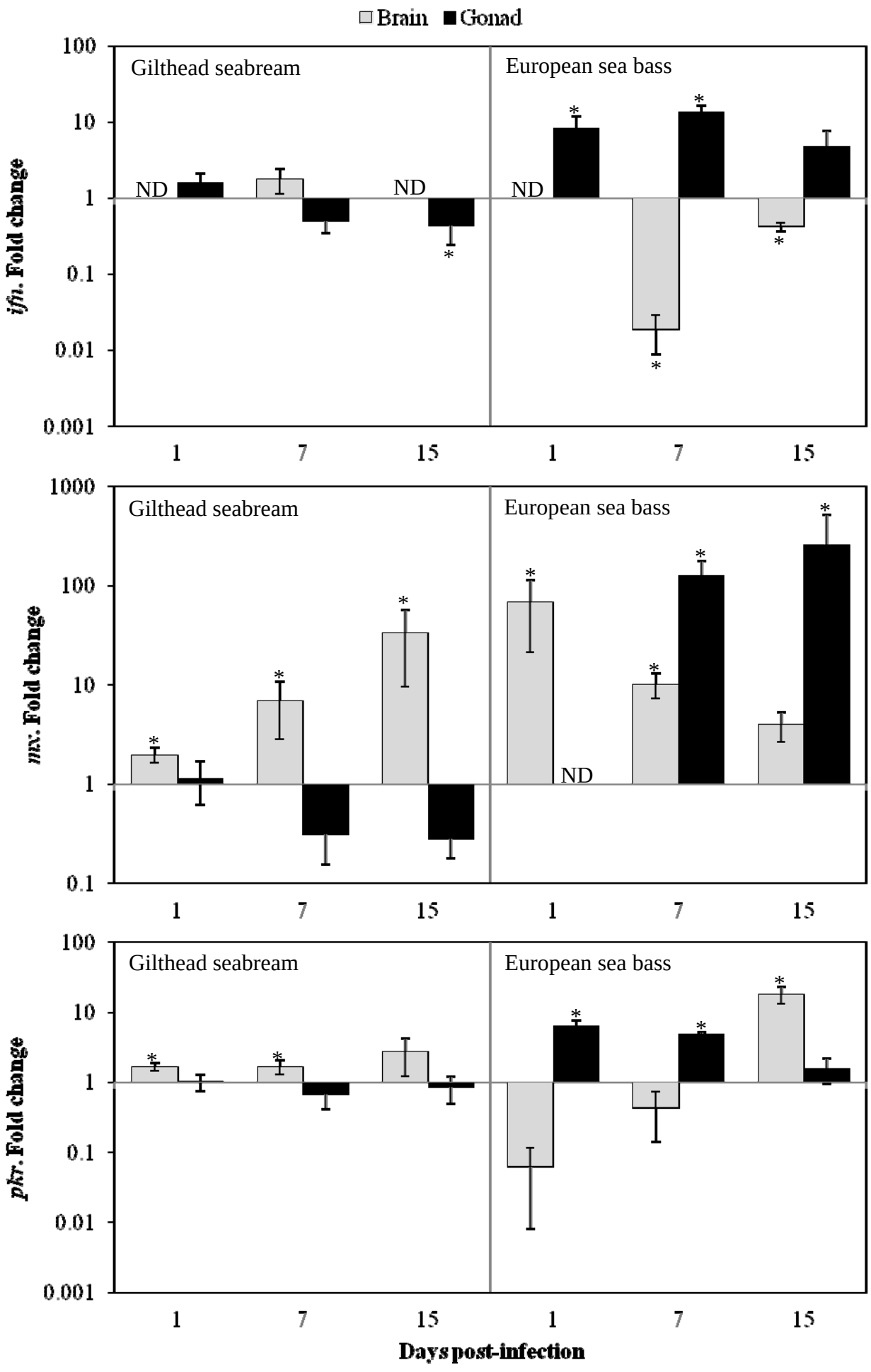
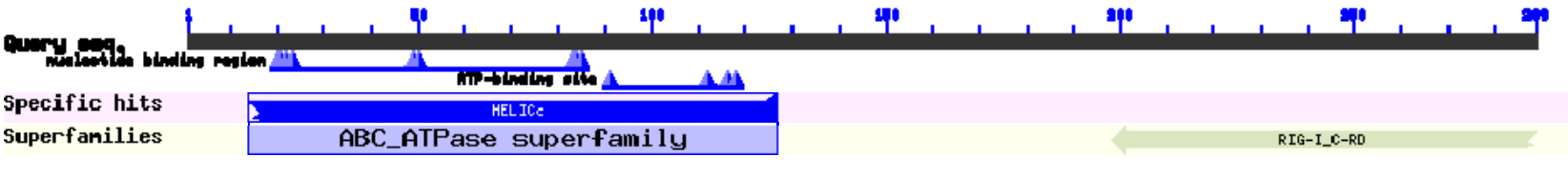
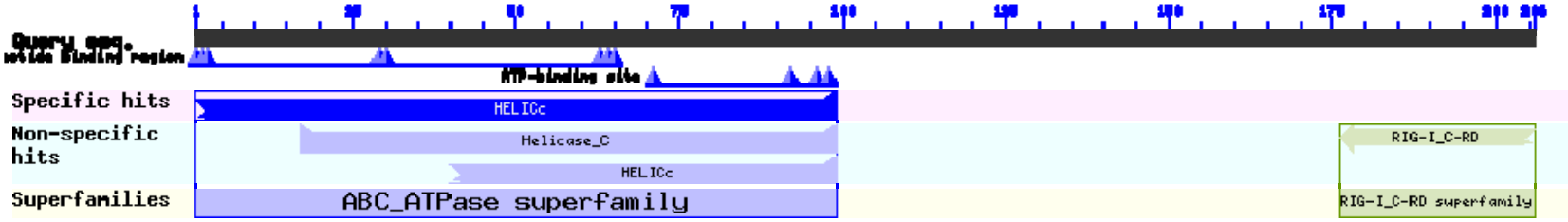
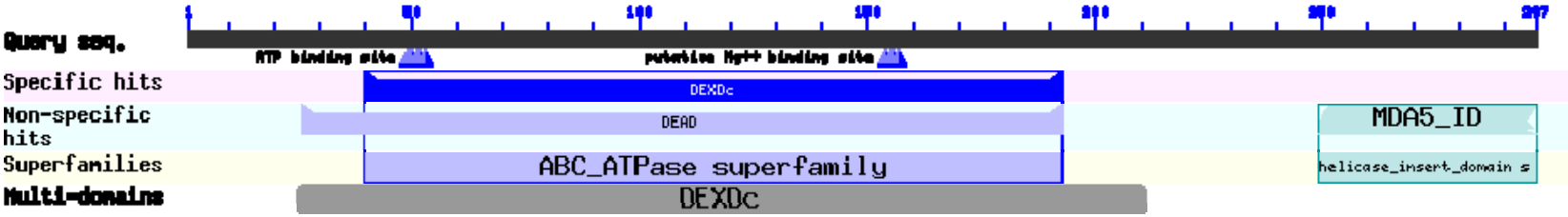
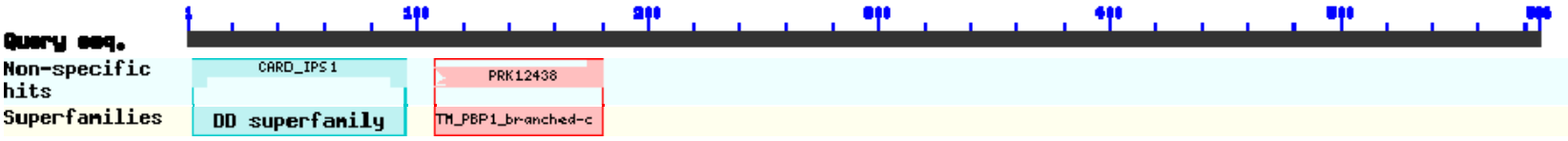
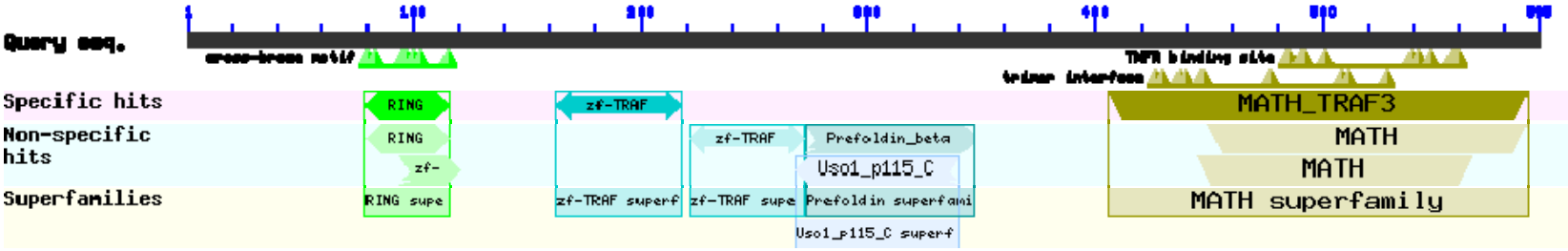
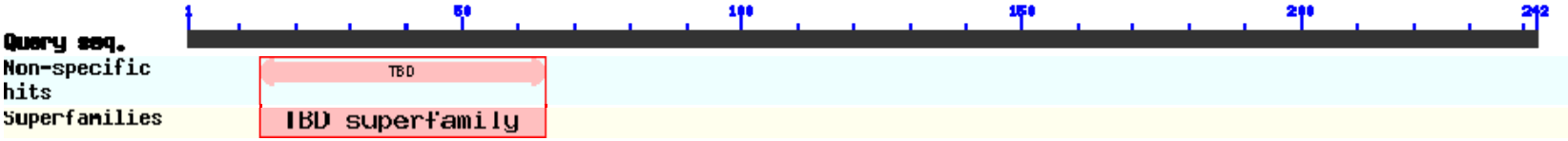
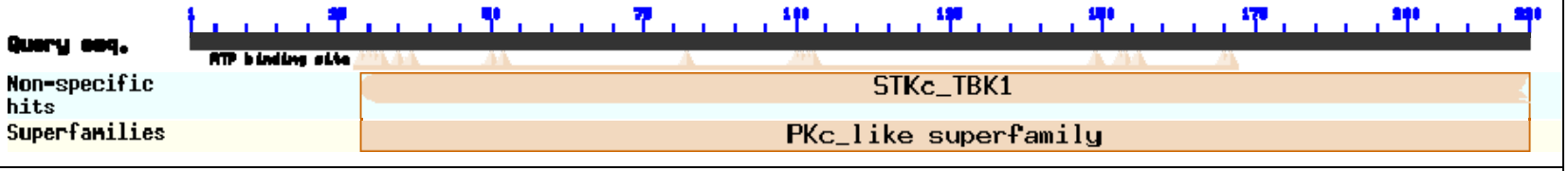
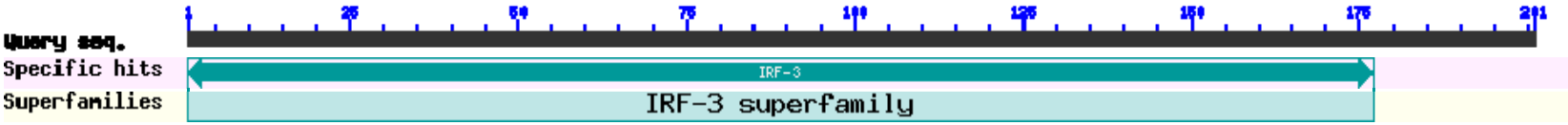
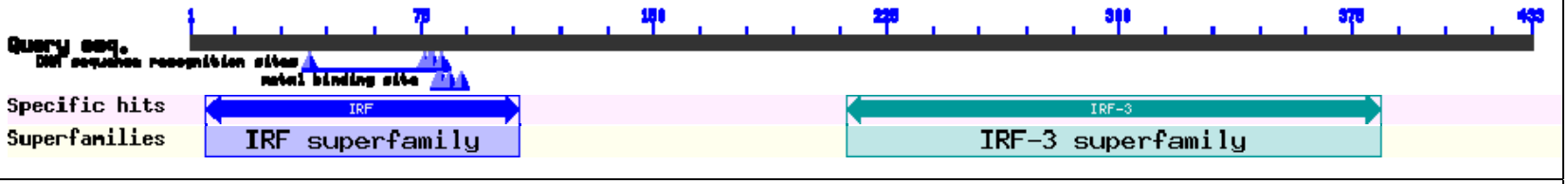
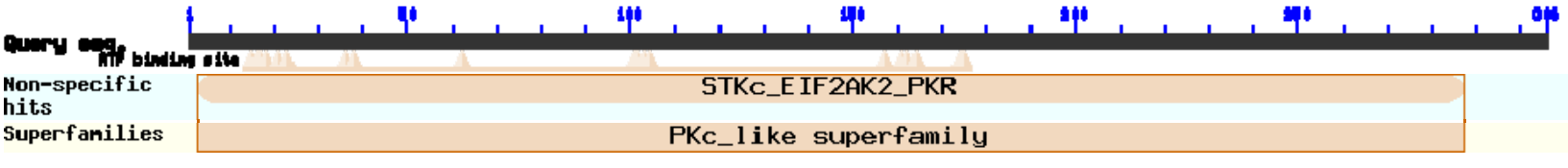

Brain
Gonad
Gilthead seabream
European sea bass
ND
ND
ND
ifn. Fold change
100
10
1
0.1
0.01
0.001
1
7
15
1
7
15
Gilthead seabream
European sea bass
ND
mx. Fold change
1000
100
10
1
0.1
1
7
15
1
7
15
Gilthead seabream
European sea bass
pkr. Fold change
100
10
1
0.1
0.01
0.001
1
7
15
1
7
15
Days post-infection

Figure 6

Table S1. Identification of the predicted protein domains by the GenBank databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Predicted protein	Fish species	Domains and motifs identified in the predicted protein sequences
MDA5	Gilthead seabream	
	European sea bass	
LGP2	European sea bass	
MAVS	European sea bass	

TRAF3	European sea bass	
TANK	European sea bass	
TBK1	Gilthead seabream	Sequence out of the STKc_TBK1 domain
	European sea bass	
IRF3	Gilthead seabream	
	European sea bass	

IRF7	European sea bass	
PKR	Gilthead seabream	
	European sea bass